

# *Bacillus subtilis* RecA with DprA–SsbA antagonizes RecX function during natural transformation

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## ABSTRACT

*Bacillus subtilis* DprA and RecX proteins, which interact with RecA, are crucial for efficient chromosomal and plasmid transformation. We showed that RecA, in the rATP-Mg<sup>2+</sup> bound form (RecA-ATP), could not compete with RecX, SsbA or SsbB for assembly onto single-stranded (ss)DNA, but RecA-dATP partially displaced these proteins from ssDNA. RecX promoted reversible depolymerization of preformed RecA-ATP filaments. The two-component DprA–SsbA mediator reversed the RecX negative effect on RecA filament extension, but not DprA or DprA and SsbB. In the presence of DprA–SsbA, RecX added prior to RecA-ATP inhibited DNA strand exchange, but this inhibition was reversed when RecX was added after RecA. We propose that RecA nucleation is more sensitive to RecX action than is RecA filament growth. DprA–SsbA facilitates formation of an active RecA filament that directly antagonizes the inhibitory effects of RecX. RecX and DprA enable chromosomal transformation by altering RecA filament dynamics. DprA–SsbA and RecX proteins constitute a new regulatory network of RecA function. DprA–SsbA contributes to the formation of an active RecA filament and directly antagonizes the inhibitory effects of RecX during natural transformation.

## INTRODUCTION

Natural transformation, which is activated through a dedicated transcription program, is an important horizontal gene transfer mechanism for acquisition of genetic diversity, as well as for the spread of antibiotic resistance, metabolic pathways or pathogenicity island traits (1,2).

Natural transformation allows efficient uptake of exogenous DNA, followed by its internalization as linear single-stranded (ss)DNA; it is then integrated onto the homologous recipient chromosome (chromosomal transformation) or established as an episome if there is no homology with the recipient. This incoming DNA must encode an autonomous replication origin (plasmid transformation) (3).

To better understand the molecular basis of natural transformation, we used *Bacillus subtilis* cells as a model. Natural competence is induced in a subset of these bacteria by starving cells of critical nutrients (3–5). DNA replication is halted in the competent subpopulation, expression is induced of *recA*, *ssbA* (*ssbEco* counterparts) and competence-specific *dprA* and *ssbB* among many other genes, and the competence uptake machinery is built at one of the cell poles (reviewed in 3,5,6). Cytosolic RecA, SsbB and DprA proteins, which interact physically with one another, as well as RecX, which interacts physically with RecA, localize transiently to the cell pole and co-localize with the DNA uptake apparatus (7,8); the location of the essential SsbA protein remains unknown.

The DNA uptake machinery processes exogenous double-stranded (ds)DNA, and takes up and internalizes linear ssDNA in a nonpolar fashion (reviewed in 3,5). The fate of the internalized ssDNA during transformation is poorly understood. Given their much higher affinity for ssDNA than DprA or RecA, either of the single-stranded binding (SSB) proteins (SsbA, SsbB) must be the first to bind incoming ssDNA as soon as it leaves the entry channel (9,10). At the entry pole and with the help of accessory proteins, RecA polymerizes on the internalized ssDNA. RecA then forms threads (filamentous structures) on the incoming ssDNA from the entry channel to the cell nucleoid (3,5). Finally, in the presence of accessory factors, RecA searches efficiently for a unique homologous sequence and promotes DNA strand exchange (DSE) in a reaction that requires nucleotide cofactor binding and hydrolysis *in vivo* (reviewed in 11,12–14).

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Lack of RecA blocks ( $\sim 10\,000$ -fold) chromosomal transformation. The accessory factors that assist RecA can be divided into two broad classes, those that act before and those that act during homology search and DSE. Some of these factors are specific for genetic recombination, for recombinational repair, or for both (3). During genetic recombination, the accessory proteins that act before homology search can again be divided into those that promote (DprA, RecO[R]), limit RecA (SsbA, SsbB) or activate RecA nucleation to catalyze DSE in the presence of adenosine triphosphate (ATP) (SsbA and DprA or RecO[R], two-component mediators (9,10,15–17). The proteins that act during homology search are RecX and RecU (3). Except for the essential SsbA, of all RecA accessory factors only DprA and RecX have a crucial role in chromosomal transformation on an otherwise wild-type (wt) background (3). Indeed, lack of RecX or DprA decrease chromosomal transformation by  $\sim 200$ - and  $\sim 70$ -fold, respectively, whereas lack of accessory factors that also contribute to recombinational repair, such as AddAB (RecBCD<sub>Eco</sub> counterpart), RecF, RecR, RecO, RecU or PcrA (RecF17) reduces chromosomal transformation by  $<3$ -fold in otherwise wt competent cells (3,18).

Little is known about these ubiquitous DprA and RecX proteins. DprA has two distinct activities, (i) to facilitate RecA nucleation and filament growth on SsbA-coated ssDNA and promote RecA-mediated DSE, crucial for chromosomal transformation, and (ii) to mediate ssDNA annealing of complementary strands coated by SsbA or SsbB during plasmid transformation (9,10,15,19). RecX, which shares limited identity with RecX<sub>Eco</sub>, co-localizes mainly with the RecA threads (8). In the absence of RecX, the metastable reversible RecA threads are more stable (8), which suggests that RecX modulates the fate of RecA ‘filament’ growth.

Uncontrolled recombination leads to genomic instability. RecA nucleoprotein filaments (NPFs) assemble in kinetically distinct phases *in vitro*, and are finely tuned by a range of regulatory mechanisms; the factors that control recombinase disassembly are only partially understood (reviewed in 13,20,21). RecA<sub>Eco</sub> is the best characterized recombinase; in its rATP·Mg<sup>2+</sup>-bound form, RecA<sub>Eco</sub>·ATP binds and slowly nucleates on ssDNA, followed by rapid filament assembly; filament disassembly then links to end-dependent ATP hydrolysis, with both processes occurring primarily in the 5'  $\rightarrow$  3' direction (reviewed in 11,12–14). During recombinational repair, regulation of RecA<sub>Eco</sub> filament assembly requires RecFOR<sub>Eco</sub> to stimulate nucleation and filament growth on SSB<sub>Eco</sub>-coated ssDNA until a dynamic equilibrium is reached between binding and dissociation (22–26). Through direct interaction with RecA<sub>Eco</sub>, RecX<sub>Eco</sub> then impedes RecA<sub>Eco</sub>·ATP filament extension, leading to net filament disassembly (27–30). In contrast, RecF<sub>Eco</sub> modulates RecA<sub>Eco</sub> assembly by antagonizing the RecX<sub>Eco</sub> negative effect, specifically during the RecA<sub>Eco</sub> extension phase, by direct RecF<sub>Eco</sub>–RecX<sub>Eco</sub> interaction (31).

How RecA assembly and disassembly are regulated during genetic recombination is little understood. RecA<sub>Eco</sub> shows some differences with recombinase from natural competent bacteria. In their ATP-bound form, these recombinases can nucleate on protein-free ssDNA, but they cannot catalyze DSE in the absence of accessory fac-

tors (inactive RecA) (10,32–34). Unlike RecA<sub>Eco</sub>·ATP (11–13) or RecA·dATP (32,35), RecA·ATP cannot nucleate or polymerize in the SsbA- or SsbB-ssDNA complexes (10,16,17,36). The presence of DprA reverses the negative effect of SsbA or SsbB on RecA filament growth, and DprA–SsbA are necessary and sufficient to activate RecA·ATP to catalyze bidirectional DSE, with greater efficiency in the 5'  $\rightarrow$  3' direction (10,17). The RecA NPFs formed in the presence of SsbB and DprA nonetheless cannot engage RecA in DSE (10). The role of the RecX protein in RecA NPF fate is poorly understood, and the factor(s) that reverse(s) RecX activity during natural chromosomal transformation is(are) unknown.

To understand how RecX regulates RecA activities, and describe the molecular mechanisms that control these modulations, we studied RecA nucleation and NPF extension on ssDNA in the presence of RecX using RecA (d)ATP hydrolysis assays, single-ssDNA manipulation using magnetic tweezers and RecA-mediated ATP-dependent DSE assays. RecX prevented nucleation and filament growth, and promoted active depolymerization of preformed RecA NPF. DprA–SsbA load RecA onto ssDNA and promotes RecA activation. Activated RecA filaments reversed the RecX negative effect. These results provide clear evidence that DprA–SsbA decrease the free energy cost for RecA binding to ssDNA, and increase binding affinity and stability of the RecA filament. This active filament antagonizes RecX inhibitory effects on RecA polymerization. RecA nucleation remained sensitive to RecX, which indicated that DprA–SsbA did not antagonize RecX directly. In the absence of DNA homology, as in natural plasmid transformation, RecX promotes RecA disassembly and DprA anneals the SsbA-coated complementary strands to reconstitute a circular replicon.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and natural transformation

Unless stated otherwise, genes and products are from *B. subtilis*. The nomenclature used to denote the origin of proteins from other bacteria is based on genus and species [e.g. *Escherichia coli* RecA is referred to as RecA<sub>Eco</sub>]. *Escherichia coli* BL21(DE3)[pLysS] cells bearing pCB722 *ssbA* (36), pCB777 *ssbB* (9), pCB888 *dprA* (15) or pCB936 *recX* (8) were used to overproduce the SsbA, SsbB, DprA or RecX proteins. *Escherichia coli* XL1-ble cells bearing pGEM3 Zf(+) were used to purify the 3199-nt pGEM3 Zf(+) ssDNA and 3199-bp pGEM3 Zf(+) dsDNA. All *B. subtilis* strains listed (Supplementary Table S1) were isogenic with BG214 (8,15). The null *recX* mutation ( $\Delta recX$ ) was mobilized by SPP1-mediated transduction into the BG1163 ( $\Delta dprA$ ) to render the isogenic BG1609 strain. BG214 cells bearing pBT61 *recA* were used to overproduce RecA (37).

For DNA transformation experiments, *B. subtilis* competent cells were transformed with 100 ng of *B. subtilis* SB19 chromosomal DNA (to *met*<sup>+</sup>) or pUB110 plasmid DNA (to neomycin resistance; Nm<sup>R</sup>). Chromosomal transformants were plated on minimal medium lacking methionine, and plasmid transformants on LB agar plates containing Nm (5  $\mu$ g ml<sup>−1</sup>). Transformation efficiency was normalized to

total viable LB-plated cells, and values obtained were normalized to those for *rec*<sup>+</sup> cells.

### Enzymes, reagents, protein and DNA purification

All chemicals used were analytical grade. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was from Calbiochem. ATP, dATP or ATP $\gamma$ S were from Sigma. DEAE, Q- and SP-Sepharose were from GE Healthcare, hydroxyapatite from BioRad and phosphocellulose from Whatman.

SsbA (18.7 kDa), SsbB (12.4 kDa), DprA (32.7 kDa) and RecA (38.0 kDa) proteins were purified as described (15,35,36). RecX (31.2 kDa) or RecX342 $\Delta$ C30 (27.3 kDa) proteins were expressed and purified; in brief, the cell pellet was resuspended in buffer A (50 mM Tris-HCl pH 7.4, 15% glycerol) containing 100 mM NaCl, passed through a French press and centrifuged to remove debris. RecX or RecX342 $\Delta$ C30 in the soluble fraction was precipitated with polyethylenimine (PEI) to a final concentration of 0.25% ( $A_{260} \sim 120$ ) and the mixture centrifuged (30 000 *g*, 30 min, 4°C). RecX or RecX342 $\Delta$ C30 were resuspended from the pellet in buffer A containing 400 mM NaCl, precipitated (70% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and resuspended in the same buffer lacking NaCl. RecX or RecX342 $\Delta$ C30 were loaded onto a phosphocellulose column equilibrated with buffer A containing 150 mM NaCl, eluted with buffer A containing a 400–800 mM NaCl gradient and protein-containing fractions were diluted to 100 mM NaCl. RecX or RecX342 $\Delta$ C30 was loaded onto a Blue Sepharose column equilibrated with the same buffer. RecX or RecX342 $\Delta$ C30 were eluted with a 200–600 mM NaCl linear gradient. The peak fractions containing RecX or RecX342 $\Delta$ C30 were loaded onto a SP-Sepharose column and stored in buffer A containing 50% glycerol at –80°C. All proteins were purified to >98% homogeneity and sequenced by automated Edman degradation.

The molar extinction coefficients for SsbA, SsbB, DprA, RecX, RecX342 $\Delta$ C30 and RecA were calculated as 11 400; 13 000; 45 500, 16 400, 13 400 and 15 200 M<sup>–1</sup> cm<sup>–1</sup>, respectively, at 280 nm, as described (38). Protein concentration was determined using these molar extinction coefficients. RecA, RecX and RecX342 $\Delta$ C30 are expressed as moles of monomeric, DprA as dimeric, and SsbA and SsbB as tetrameric proteins. Duplex DNA from 3199-bp pGEM3 Zf(+) and ssDNA from 3199-nt pGEM3 Zf(+) were purified as reported (38). DNA concentrations were established using molar extinction coefficients of 8780 and 6500 M<sup>–1</sup> cm<sup>–1</sup> at 260 nm for ssDNA and dsDNA, respectively, and are expressed as moles of nt.

Protein concentrations are expressed in the text as stoichiometric ratios relative to ssDNA, which is expressed as moles of nt; in figure legends, the molar concentrations of proteins and ssDNA/dsDNA are given. In this study, experiments were performed in optimal RecA conditions (10 mM magnesium acetate or 10 mM MgCl<sub>2</sub>) and SSB proteins were anticipated to occlude 65 nt, with the ssDNA wrapping around all four subunits of the tetramer (SSB<sub>65</sub>) (9). A DprA dimer should bind 30–40 nt (15), and a RecA monomer should bind 3 nt (39). The RecX binding size is unknown.

### RecA (d)ATP hydrolysis assays

The ssDNA-dependent dATP or ATP [(d)ATP] hydrolysis activity of RecA protein was assayed via a coupled spectrophotometric enzyme assay as described (9,23). The rate of ssDNA-dependent RecA-mediated (d)ATP hydrolysis and lag times were measured in buffer B (50 mM Tris-HCl pH 7.5, 1 mM DTT, 80 mM NaCl, 10 mM magnesium acetate, 50  $\mu$ g/ml bovine serum albumin, 5% glycerol) containing 5 mM (d)ATP and incubated (30 min, 37°C) as described (9). The control reaction with RecX (1 RecX /33-nt) alone did not show (d)ATP hydrolysis activity compared with the mock reaction in the absence of RecX protein, suggesting that the ATP hydrolysis observed can be attributed solely to RecA protein. None of the SSB proteins showed (d)ATP hydrolysis activity. The order of addition of 3199-nt pGEM3 Zf(+) ssDNA (10  $\mu$ M in nt) and of purified proteins are indicated in the text. Proteins used were RecA (800 nM), SsbA or SsbB (300 nM), DprA (100 nM) and RecX (concentrations indicated in the figure). Data from (d)ATP hydrolysis were converted to [(d)ADP] and plotted as a function of time, as described (9). Lag time, which represents the delay in reaction progress relative to a theoretical reaction curve, was derived from the time intercept of a linear regression line fit to the steady state portion of data in (d)ATP hydrolysis assays (9,23).

### RecA-mediated DNA strand exchange

Standard reactions containing 3199 bp KpnI-cleaved pGEM3 Zf(+) dsDNA (20  $\mu$ M in nt) and the homologous circular 3199-nt ssDNA (10  $\mu$ M in nt) were pre-incubated with SsbA (300 nM), DprA (60 nM) and increasing concentrations of RecX or RecX342 $\Delta$ C30 (0.3, 0.7, 1.5, 3, 6, 12 and 25 nM) (denoted as first [1°] RecX or RecX342 $\Delta$ C30) or fixed RecA (800 nM) (first [1°] RecA) in buffer A containing 5 mM ATP (5 min, 37°C). Then, a fixed RecA (1° RecX or RecX342 $\Delta$ C30) or variable RecX or RecX342 $\Delta$ C30 (1° RecA) concentration was added, and the reaction was incubated (60 min, 37°C). An ATP regeneration system (8 U ml<sup>–1</sup> creatine phosphokinase and 8 mM phosphocreatine) was included in the recombination reaction. After the reaction took place, samples were deproteinized and fractionated by 0.8% agarose gel electrophoresis (AGE) with ethidium bromide (40,41). The signal of the DNA substrates and products was quantified from gels using a Geldoc (BioRad) system (35).

### Single molecule analyses

An in-house-built vertical magnetic tweezers system (42,43) was used for all single-ssDNA manipulation experiments in this study. A disturbance-free, rapid solution-exchange method using microwell assays (44) was combined with the magnetic tweezers system to monitor ssDNA extension dynamics during/after protein solution change. The 572-nt ssDNA was prepared in the flow channel as described (45–47). Protein or protein mixtures were then introduced to the ssDNA tethers in a solution volume of  $\sim 200$   $\mu$ l unless otherwise stated (channel volume =  $40 \pm 10$   $\mu$ l). Experiments were performed in the standard RecA reaction, in buffer C (20 mM Tris-HCl pH 7.4, 10 mM MgCl<sub>2</sub>, 50 mM NaCl)



at 23°C. In all reactions containing RecA, 1 mM (d)ATP regeneration system (see above) were added, except where otherwise stated. For additional details of single-ssDNA manipulation including force calibration, ssDNA generation and extension measurement, disturbance-free rapid solution exchange method and step-finding algorithm, see previous publications (42–45,47).

## RESULTS

### RecX and DprA regulate RecA during genetic recombination

The accessory proteins DprA and SsbA recruit RecA to ssDNA, stimulate RecA polymerization on incoming ssDNA, promote RecA-mediated DSE, crucial for chromosomal transformation and mediate ssDNA annealing of complementary strands coated by SsbA during plasmid transformation (10). With the help of RecX, a RecA NPF with an effective length searches efficiently for a unique homologous sequence (a crucial step for chromosomal transformation in the haploid genome of natural competent cells) and helps to dislodge RecA from incoming ssDNA (8). The transformation of natural plasmids, which share no notable degree of identity (>30 nt) with the recipient genome, is RecA-independent, but requires DprA and RecX (8,15,48).

The chromosomal and plasmid transformation defects of  $\Delta recA$ ,  $\Delta recX$  and  $\Delta dprA$  mutant strains have been analyzed in various contexts, and were re-evaluated here for direct comparison on a prophage-free isogenic background (Supplementary Table S1). Inactivation of RecA abolished chromosomal transformation, but only marginally impaired plasmid transformation, whereas the absence of DprA or RecX decreased both chromosomal and plasmid transformation (Supplementary Table S1).

Competent cells that lacked RecA and DprA were blocked for both chromosomal and plasmid transformation, whereas lack of RecA and RecX blocked chromosomal and suppressed the plasmid transformation defect (Supplementary Table S1). We hypothesized that (i) a RecA filament formed on heterologous ssDNA (plasmid ssDNA) should be unproductive and must be disassembled (49); (ii) in the absence of RecX, a long-lived RecA filament that assembled on the plasmid ssDNA is deleterious for plasmid transformation in the absence of DNA homology with the recipient; and (iii) in the absence of RecA, RecX-mediated disassembly of the RecA NPF is not required and DprA can catalyze the annealing of complementary strands coated by SsbA or SsbB to reconstitute the circular replicon during plasmid transformation (15). This is consistent with the observation that RecX, which interacts physically with RecA (8), is necessary to modulate the RecA NPF half-life (RecA threads) during chromosomal transformation. We constructed a null *recX dprA* double mutant strain and examined chromosomal and plasmid transformation efficiency. Competent cells lacking RecX and DprA were blocked for chromosomal transformation, but plasmid transformation efficiency was comparable to the more deficient single mutant (Supplementary Table S1). RecX probably works in concert with RecA during chromosomal and with DprA during plasmid transformation in an otherwise wt background. The mechanism by which RecX acts and how RecX activity is regulated during genetic recombination nonetheless remained elusive.

### RecX inhibits RecA-catalyzed ATP hydrolysis

To characterize the role of RecX in RecA nucleation and polymerization onto ssDNA, we purified the proteins and used the kinetics of RecA-mediated hydrolysis of ATP as an indirect readout (35). RecA·ATP (1 RecA monomer/12 nucleotides [nt]) nucleation and polymerization onto ssDNA showed a monophasic shape and hydrolyzed ATP at a catalytic rate constant ( $K_{cat}$ ) of  $9.3 \pm 0.2 \text{ min}^{-1}$  (Figure 1A), similar to data for comparable experimental conditions (10,16).

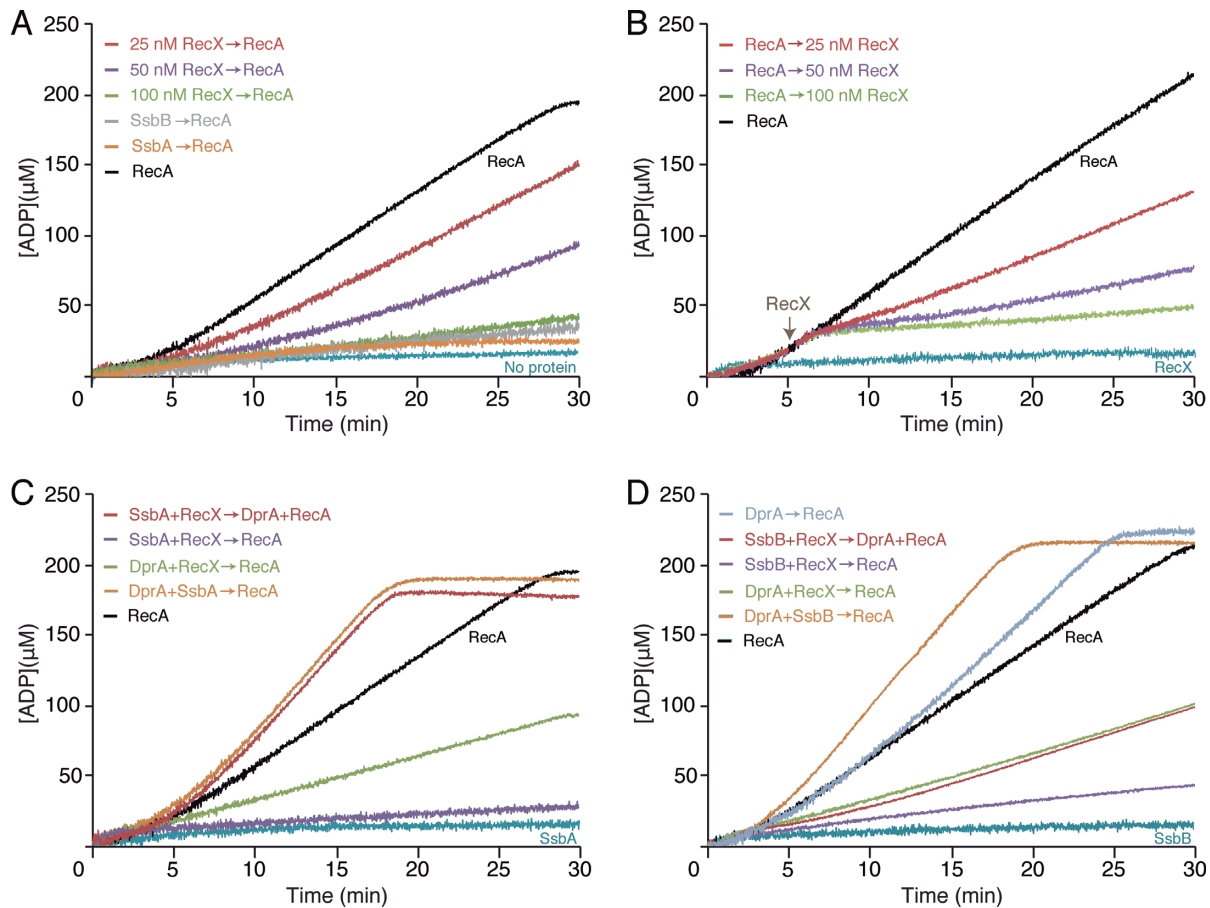
Limiting RecX (1 RecX monomer/400–200 nt) reduced ATP hydrolysis when added before ( $K_{cat}$  7.4 and  $5.5 \text{ min}^{-1}$ , respectively) or after RecA ( $K_{cat}$  5.8 and  $3.1 \text{ min}^{-1}$ , respectively) (Figure 1A and B). Subsaturation concentrations (1 RecX/100 nt) inhibited ATP hydrolysis ( $K_{cat} < 2 \text{ min}^{-1}$ ) when added before or after RecA (Figure 1A and B). RecX<sub>Eco</sub>, *Neisseria gonorrhoeae* RecX (RecX<sub>Ngo</sub>) or *Mycobacterium tuberculosis* RecX (RecX<sub>Mtu</sub>) similarly inhibit the ATPase activity of their cognate RecA proteins in the presence of naked ssDNA (27,50,51).

At saturating SSB concentrations (1 SSB tetramer/33 nt), SsbA or SsbB inhibited RecA·ATP nucleation and filament growth on ssDNA to background levels ( $K_{cat} < 2 \text{ min}^{-1}$ ), which were similar to RecX alone (1 RecX/100 nt) (Figure 1A and B). Assay sensitivity in the presence of ATP did not permit observation of the SSB and RecX protein effect on RecA-mediated ATP hydrolysis (Figure 1C and D).

### RecA·dATP partially reverses RecX inhibitory effects

RecA hydrolyzes dATP ~50% more rapidly than ATP (36). RecA affinity for ssDNA is greater when ATP is replaced with dATP, and RecA·dATP can partially displace SsbA or SsbB from ssDNA (Supplementary Figure S1A) (9,10). To test whether RecA·dATP competes with RecX and whether saturating SSB concentrations reverse the RecX negative effect on RecA assembly on ssDNA, we replaced ATP with dATP as nucleotide cofactor. After an ~4 min lag, RecA hydrolyzed dATP at near the previously observed  $K_{cat}$  of  $17.8 \pm 0.2 \text{ min}^{-1}$  (Supplementary Figure S1), similar to data reported in similar experimental conditions (9,15). When pre-incubated with ssDNA, RecX (1 RecX/33 nt) delayed RecA·dATP nucleation (lag phase ~6 min), with partial reduction of the maximal dATP hydrolysis rate ( $K_{cat}$   $12.5 \pm 0.3 \text{ min}^{-1}$ ) (Supplementary Figure S1A). RecX addition to preformed RecA filaments on ssDNA marginally reduced dATP hydrolysis for the first 3 min, and later reduced dATPase ( $K_{cat}$   $6.0 \pm 0.3 \text{ min}^{-1}$ ) (Supplementary Figure S1B).

Pre-incubation of ssDNA with SsbA or SsbB (1 SSB tetramer/33 nt) delayed nucleation and reduced or slightly impaired dATP hydrolysis ( $K_{cat}$   $13.5 \pm 0.2$  or  $15.3 \pm 0.3 \text{ min}^{-1}$ , respectively) (Supplementary Figure S1A). In a similar manner, nucleated RecA·dATP can partially displace SsbA or SsbB from ssDNA (9,15). RecX and SsbA or SsbB pre-incubated with ssDNA reduced the maximal dATP hydrolysis rate ( $K_{cat}$   $3.8 \pm 0.2$  and  $7.3 \pm 0.3 \text{ min}^{-1}$ , respectively) (Supplementary Figure S1A). When ssDNA was pre-incubated with SsbA or SsbB and RecA for 5 min, followed by RecX addition, RecA-mediated dATP hydrolysis was blocked ( $K_{cat}$   $2.0 \pm 0.2$  and  $2.7 \pm 0.3 \text{ min}^{-1}$ , respectively) (Supplementary Figure S1B). It is likely that



**Figure 1.** RecX effect on RecA nucleation and polymerization. (A) Circular 3199-nt ssDNA (10 μM in nt) was pre-incubated with increasing RecX concentrations or a fixed SSB concentration (300 nM) (5 min, 37°C) in buffer A containing 5 mM ATP. RecA (0.8 μM) was added and ATPase activity measured for 30 min. (B) ssDNA was pre-incubated with RecA (5 min, 37°C), followed by RecX and ATPase activity measured. (C and D) ssDNA was pre-incubated with indicated proteins (5 min, 37°C), followed by RecA, and ATPase activity measured. The amount of ATP hydrolyzed was calculated as described. All reactions were repeated at least three times, with similar results.

RecA·dATP was less sensitive to RecX inhibition than RecA·ATP, and that RecX and SsbA or SsbB synergistically inhibited RecA·dATP assembly onto ssDNA. Alternatively, RecA·dATP assembled in the 3' → 5' direction escapes RecX-mediated polymerization into ssDNA (see (17)).

#### RecA·ATP assembles on RecX–ssDNA–SsbA–DprA complexes more efficiently than on RecX–ssDNA–DprA or RecX–ssDNA–SsbB–DprA complexes

RecA interacts with DprA and RecX *in vivo* (7,8). By interacting with the N-terminal α-helix and the RecA DNA binding domain, DprA binds the 5'-end of the RecA filament (19,52,53), whereas by interacting with the C-terminal domain of one RecA subunit and the nucleotide-binding core of another, RecX binds the RecA filament and to the 3'-end (28,30). To test whether DprA can reverse the RecX negative effect of RecA assembly on ssDNA, we studied the kinetics of RecA-mediated hydrolysis of ATP (Figure 1C and D).

Equimolar DprA concentrations (1 DprA dimer/100 nt) partially reversed the negative effect of RecX (1 RecX/100 nt) on RecA nucleation, and the maximal ATP hydrolysis rate was partially recovered ( $K_{cat}$   $4.2 \pm 0.2$  min<sup>-1</sup>). These

data might be explained as two independent events; DprA bound to the free ssDNA region enables RecA loading on ssDNA, and RecX interaction with RecA facilitates RecA depolymerization from the ssDNA. Alternatively, an interaction between DprA and RecX might interfere with RecX function, although we were unable to detect a DprA–RecX interaction both *in vivo* (FRET analysis) or *in vitro* (pull-down experiments, protein–protein crosslinking) (data not shown).

RecA nucleated and polymerized on the DprA–ssDNA–SsbA or DprA–ssDNA–SsbB complexes more efficiently than on DprA–ssDNA complexes (Figure 1C and D). We performed ATPase assays to test whether DprA can indirectly reverse the RecX negative effect on RecA nucleation and filament formation on SSB-coated ssDNA. ssDNA was pre-incubated with saturating SsbA and equimolar DprA and RecX concentrations, followed by RecA addition. RecA nucleated and polymerized on RecX–ssDNA–SsbA–DprA complexes more efficiently than on RecX–ssDNA–DprA complexes (Figure 1C). RecA promoted ATP hydrolysis at a maximal rate ( $K_{cat}$   $15.6 \pm 0.2$  min<sup>-1</sup>), which suggested that, in the presence of the two-component mediator DprA–SsbA, DprA interaction with RecA leads

to a structural transition of the latter that in turn interacts more loosely with RecX. Alternatively, the high ATP hydrolysis rates correlate with removal of secondary structures by a Ssb protein. To test this hypothesis, we replaced SsbA with SsbB. RecA nucleated and polymerized on the RecX-ssDNA-SsbB-DprA or RecX-ssDNA-DprA complexes with similar efficiency and a low ATP hydrolysis rate ( $K_{cat} \sim 4.5 \text{ min}^{-1}$  versus  $\sim 4.2 \text{ min}^{-1}$ , respectively) (Figure 1C and D). It is likely that DprA, which reverses the negative effect of SsbB on RecA NPF formation (Figure 1A and D), in concert with SsbB were not sufficient to fully reverse the RecX negative effect on RecA nucleation and filament growth.

### RecX and SsbA inhibit stable RecA nucleation and polymerization on ssDNA

Both RecX and SsbA or SsbB synergistically inhibited RecA-catalyzed ATP hydrolysis, but DprA-SsbA reversed this inhibition (Figure 1C). To better understand the underlying mechanisms, we used a magnetic tweezers system to study the effect of SsbA or RecX on RecA-ATP nucleation and polymerization on a single 572-nt ssDNA tether formed between a coverslip and a paramagnetic bead surface in a flow channel (Figure 2A) (42,45,46). The 572-nt ssDNA can accommodate  $\sim 9$  SsbA and  $\sim 190$  RecA in the presence of 10 mM  $\text{MgCl}_2$ .

The force-extension curves of the ssDNA bound by different amounts of SsbA differed from one another (Figure 2B). At forces  $< 7$  pN, the SsbA-bound ssDNA extension was longer than naked ssDNA; at forces between 7 and 15 pN, it was shorter than naked ssDNA, but at forces  $> 18$  pN, the SsbA-bound ssDNA extension overlapped that of naked ssDNA at all SsbA concentrations tested (Figure 2B). As reported for the SSB<sub>Eco</sub> protein (45), it is likely that at low force, SsbA removes secondary structures on ssDNA; at medium force, SsbA is wrapped by ssDNA and at high force, ssDNA unspools from SsbA, which remains bound to ssDNA.

RecA was pre-incubated with SsbA and flowed them into the SsbA-bound ssDNA channel. The resulting ssDNA force-extension curves overlap with that of SsbA-coated ssDNA (Figure 2C), which indicated that the ssDNA-SsbA complex blocks RecA-ATP binding to ssDNA, and hence inhibits RecA nucleation and polymerization (Figure 1A). When RecX was incubated with ssDNA, we found no extension change of the ssDNA over a wide force range, which suggests that RecX does not interact strongly with ssDNA at these concentrations or that RecX binding does not notably perturb ssDNA conformation (Figure 2D). When a RecA-ATP and RecX mixture was incubated with the entrapped ssDNA stretched at a force of  $\sim 4.7$  pN, after a long lag phase ( $> 600$  s) we observed a dynamic extension increase/decrease over an amplitude of  $\sim 70$  nm ( $\sim 50$  RecA monomers) (Figure 2E). It is likely that after the long lag, RecA overcame the high nucleation barrier and formed unstable RecA NPF from nucleation sites in the presence of RecX. The unstable RecA NPF polymerized and depolymerized dynamically, which led to large extension fluctuations (Figure 2E). When dynamic competition between polymerization/depolymerization resulted in complete loss

of the nucleation site, it caused a new lag phase (Supplementary Figure S2A). At a force of  $\sim 11$  pN in the presence of RecX, and after a lag phase of  $\sim 180$  s, RecA-ATP polymerized on ssDNA to saturation (Figure 2G). This result indicates that a force  $> 10$  pN is sufficient to antagonize the RecX inhibitory effect, in sharp contrast to the case when RecA-ATP was incubated with ssDNA at a similar force ( $\sim 4.6$  pN) in the absence of RecX (Figure 2F, blue). After an  $\sim 350$  s lag phase (blue arrow), RecA-ATP showed stable, rapid polymerization ( $\sim 50$  nm in  $< 50$  s, purple arrow), followed by slow polymerization ( $\sim 90$  nm in  $\sim 500$  s, gray arrow). The slow polymerization process after rapid initial polymerization at low force ( $\sim 4.6$  pN) is probably due to secondary structures that impede RecA binding to ssDNA. When force was increased to  $\sim 16$  pN and the secondary structures on ssDNA were removed, RecA fully covered the ssDNA (Figure 2F, orange arrow). At a force of  $\sim 9$  pN, which removed secondary structures, the RecA-ATP nucleation lag phase was reduced to  $\sim 60$  s, followed by a rapid polymerization phase ( $\sim 30$  s), leading to fully polymerized RecA NPF (Supplementary Figure S2B) (21,46).

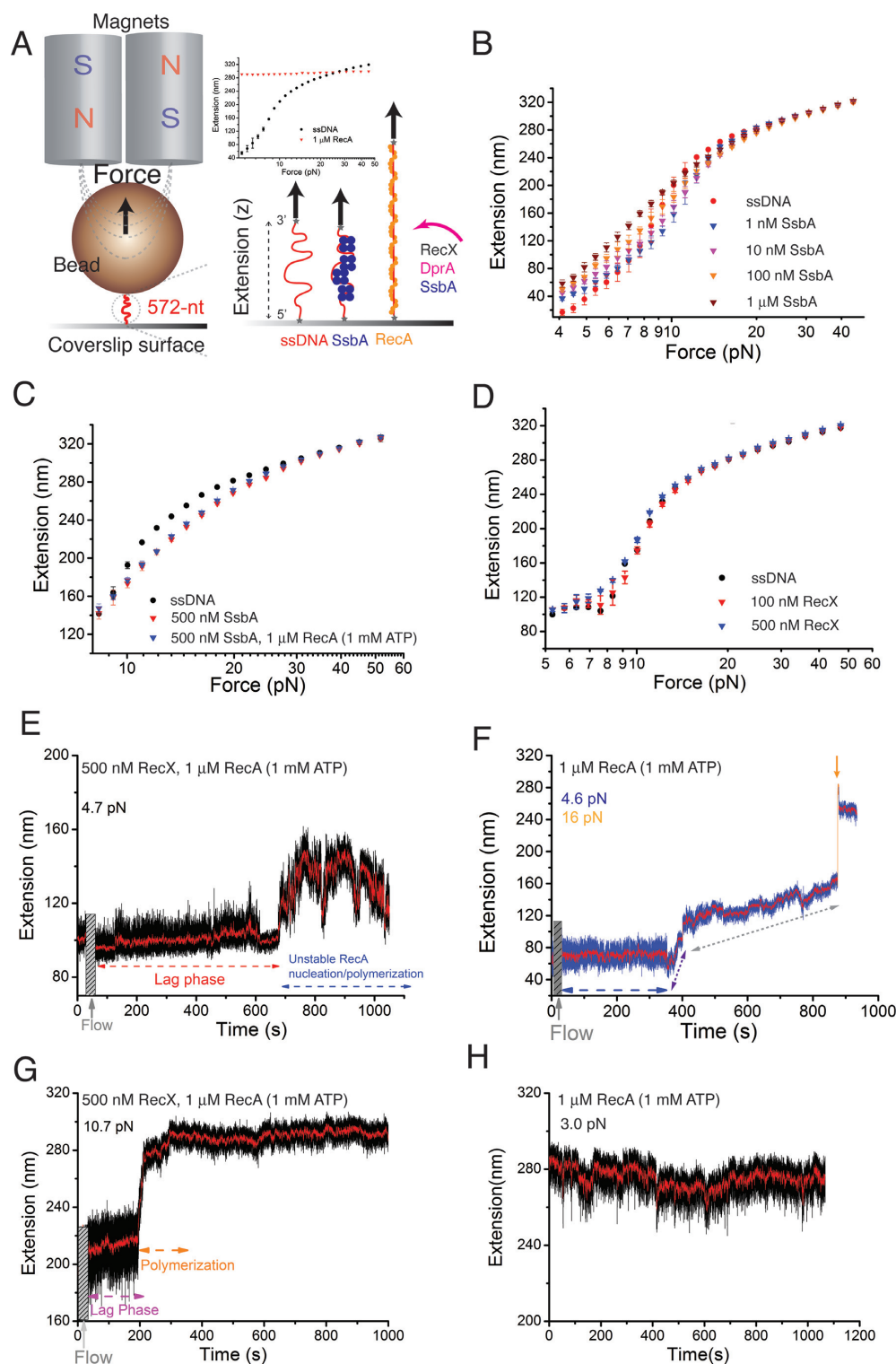
These results show that (i) SsbA outcompetes RecA binding to ssDNA and inhibits RecA nucleation on ssDNA, (ii) RecX-RecA interaction leads to unstable RecA nucleation/polymerization on ssDNA and (iii) DNA stretching by forces  $> 10$  pN facilitates a RecA-ATP transition state able to antagonize the RecX inhibitory effect on RecA nucleation.

### RecX induces RecA NPF depolymerization

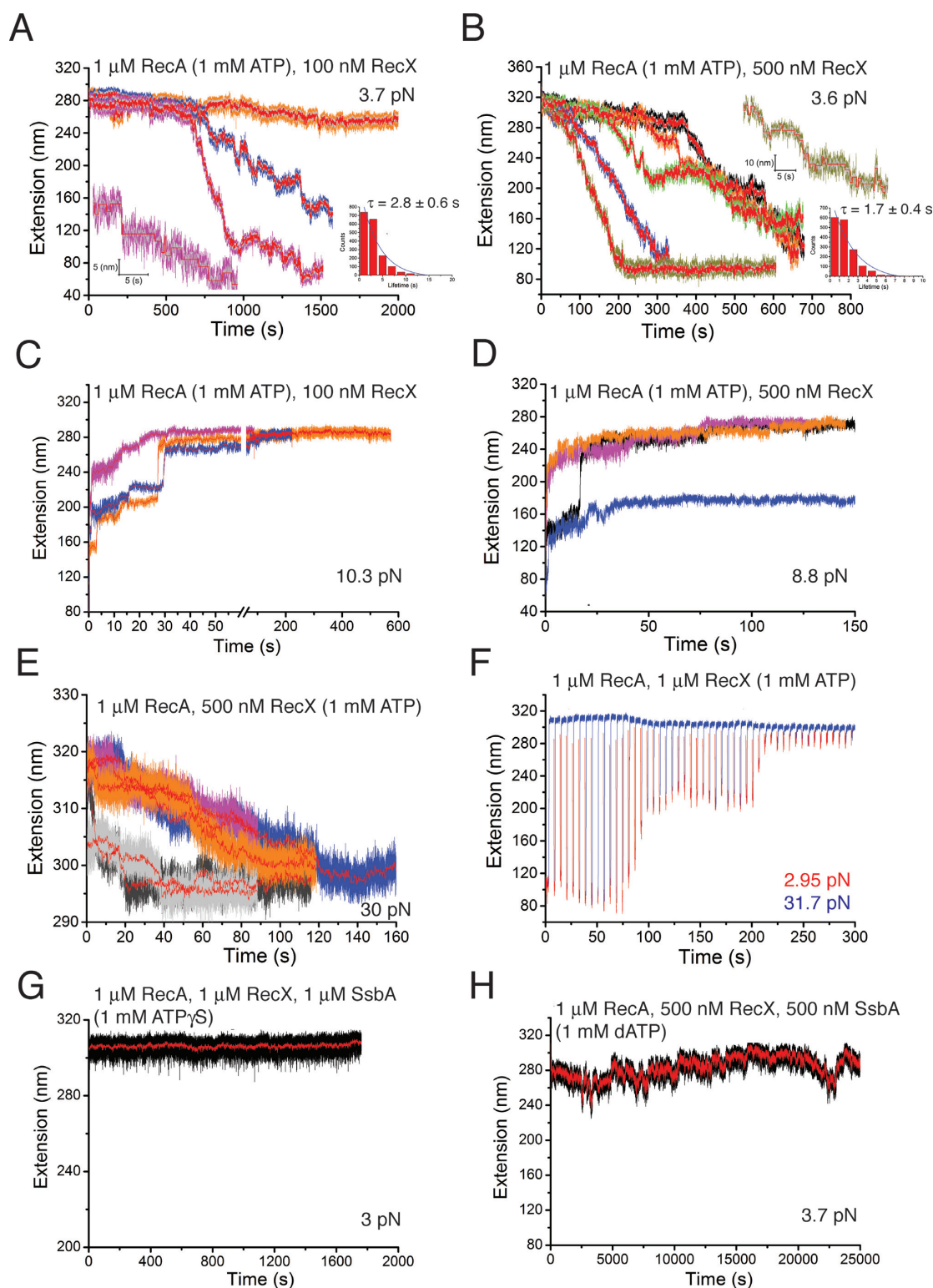
A RecA filament undergoes dynamic RecA association/dissociation presumably in both the  $5' \rightarrow 3'$  and the  $3' \rightarrow 5'$  direction, with a preference for  $5' \rightarrow 3'$  extension (17,21). To test how RecX regulates RecA filament disassembly, we analyzed extension time traces of fully polymerized RecA NPF after introduction of the RecA/RecX mixture (Figure 3A and B). Saturating RecA-ATP concentrations were polymerized to individual 572-nt ssDNA tethers at forces of  $\sim 20$  pN to allow full RecA polymerization. In the absence of RecX at low forces ( $\sim 3$  pN), the resulting RecA NPF were stable over long periods ( $> 1000$  s) (Figure 2H). When RecA-ATP and RecX (100 or 500 nM) were introduced to the fully polymerized RecA NPF and the extension of the RecA NPF tether decreased at  $\sim 3$  pN, a progressive net RecA disassembly from ssDNA was observed. RecX induced RecA depolymerization, with occasional net RecA polymerization at the RecX concentrations tested (Figure 3A and B). The characteristic lifetimes of RecA filament depolymerization (defined as the time between two adjacent depolymerization events) were estimated at  $2.8 \pm 0.6$  s (with 100 nM RecX) and  $1.7 \pm 0.4$  s (with 500 nM RecX) (Figure 3A and B). Forces  $> 8$  pN can facilitate RecA repolymerization (Figure 3C–F). These new RecA filaments again underwent depolymerization at lower forces, which suggests that RecA bound to ssDNA stretched by force can reverse the RecX negative effect on RecA nucleation and polymerization.

We observed two distinct kinetic phases of depolymerization processes, an initial lag phase of distinct duration (10–100 s) with no or very slow net depolymerization and a





**Figure 2.** RecX or SsbA inhibits stable RecA nucleation/polymerization on ssDNA. (A) Scheme showing single molecule manipulation. A 572-nt ssDNA tether was formed between surfaces of a coverslip and a paramagnetic bead inside a flow channel. By controlling the magnets, forces were applied to the ssDNA, and the extensions of ssDNA and of ssDNA bound to proteins (RecA, SsbA or RecX) were recorded. Inset, typical force-extension curves of RecA filament formed on ssDNA (red) and naked ssDNA (black) (see ‘Materials and Methods’ section). (B) Typical force-extension curves of free and SsbA-bound ssDNA with increasing SsbA concentration. (C) Typical force-extension curves of ssDNA (black) in the presence of SsbA (red) or SsbA and RecA (blue). (D) Typical force-extension curves of ssDNA in the presence of RecX. (E) Typical extension/time trace of ssDNA after introduction (gray arrow) of a RecX and RecA mixture at a low force of ~4.7 pN. (F) Typical extension/time trace of ssDNA after introduction (gray arrow) of a RecX and RecA mixture at a low force (~4.6 pN; blue), with short duration of a higher force (~16 pN; orange arrow). (G) Typical extension/time trace of ssDNA after introduction (gray arrow) of a RecX and RecA mixture at a higher force of ~10.7 pN. (H) Typical extension/time trace of ssDNA–RecA filament with RecA in solution at a low force of ~3 pN. Lag phase lines in E–G are the 40-point smooth of the raw data (black/blue).



**Figure 3.** RecX induced net depolymerization of preformed RecA filament. (A and B) Typical extension time traces of net depolymerization of RecA filaments in the presence of two RecX concentrations and RecA at  $\sim 3$  pN. Gray lines indicate the 40-point smooth of raw data (colored), red lines are stepwise traces using a previously developed step-finding algorithm (see 'Materials and Methods' section). The inserted extension time traces are zoomed examples of the stepwise traces. Inserted histograms are the depolymerization lifetime distribution determined by the algorithm. Blue lines in the histograms are exponential decay fitting curves, from which the lifetime characteristics were determined. (C and D) Typical extension time traces of partially depolymerized RecA filaments after increase to  $\sim 9$  or  $10$  pN in the presence of different amounts of RecX and RecA. (E) Typical extension time traces of partially depolymerized RecA filament after increasing the force to  $\sim 30$  pN in the presence of RecX. (F) Typical extension time traces of partially depolymerized RecA filaments cycling between  $\sim 3$  pN (red) and  $> 32$  pN (blue) in the presence of RecA and RecX. (G and H) Typical extension time traces of preformed RecA filament in the presence of RecX, SsbA and ATP $\gamma$ S (G) or dATP (H) at  $\sim 3$  pN.



strikingly rapid net depolymerization phase. In some time traces, progressive depolymerization paused, then resumed after different times (10–100 s); others did not show the initial lag phase, but it might have a very short lag phase (Figure 3A and B).

We examined the effect of force on RecA filament stability in the presence of RecX. At ~9–10 pN force, we observed RecA-ATP polymerization at two RecX concentrations (Figure 3C and D). Extension increased in both the conditions, with two kinetic types, large abrupt stepwise extension jumps and slow gradual extension increases. The large abrupt stepwise RecA-induced extension jumps were not observed in the absence of RecX. High force facilitates RecA filament repolymerization in the presence of RecX (Figure 3C–F and Supplementary Figure S3C) or RecX and SsbA (Supplementary Figure S3D).

RecX-induced RecA filament depolymerization is ATP hydrolysis dependent. RecA disassembly was not observed when ATP $\gamma$ S replaced ATP in the presence of RecA and RecX (alone or with SsbA) (Figure 3G and Supplementary Figure S2C). At low forces, replacing ATP with dATP also stabilized the pre-assembled RecA filament in the presence of RecX and SsbA (Figure 3H and Supplementary Figure S2D), which suggests that stretching the ssDNA or incubating RecA with dATP would facilitate a RecA transition state that can reverse the RecX effect.

#### DprA reverses the RecX and SsbA inhibitory effects on RecA filament formation

DprA and SsbA promoted RecA-catalyzed ATP hydrolysis in the presence of RecX (Figure 1C). Consistent with this, stoichiometric amounts of DprA reversed the inhibitory effects of RecX and SsbA at low force, leading to stable RecA filament polymerization on ssDNA after an ~100 s lag phase (Figure 4A). DprA also stabilized the preformed RecA filament in the presence of stoichiometric amounts of RecX over ~1000 s (Figure 4B), which antagonizes the RecX inhibitory effects (Figure 3A–B). These results and those in the previous section provided evidence that DprA interacting with the 5'-end of a RecA filament facilitates filament growth, even in the presence of RecX.

#### Nucleated RecA-ATP on the SsbA-ssDNA-DprA complexes reverses the RecX inhibitory effect on RecA-mediated strand exchange

DprA facilitates spontaneous SsbA sliding along ssDNA, distorts the ssDNA structure and recruits RecA onto SsbA-coated ssDNA (10). The DprA-SsbA mediator is necessary and sufficient to activate RecA-ATP to catalyze DSE (see 10,17). We thus proposed that DprA-SsbA, through direct DprA-RecA interaction enables RecA to overcome the RecX inhibitory effect on RecA-ATP-mediated DSE. To test this hypothesis, we analyzed the RecX effect on RecA-mediated three-strand exchange reaction (Figure 4C and D).

RecA-ATP neither displaced SsbA or SsbB from ssDNA (Figure 1A) nor catalyzed DSE (10); DprA (1 DprA/160 nt) and SsbA (1 DprA/33 nt) were thus added to the DSE reaction. A constant amount of DprA-SsbA and a variable RecX concentration were pre-incubated with ssDNA

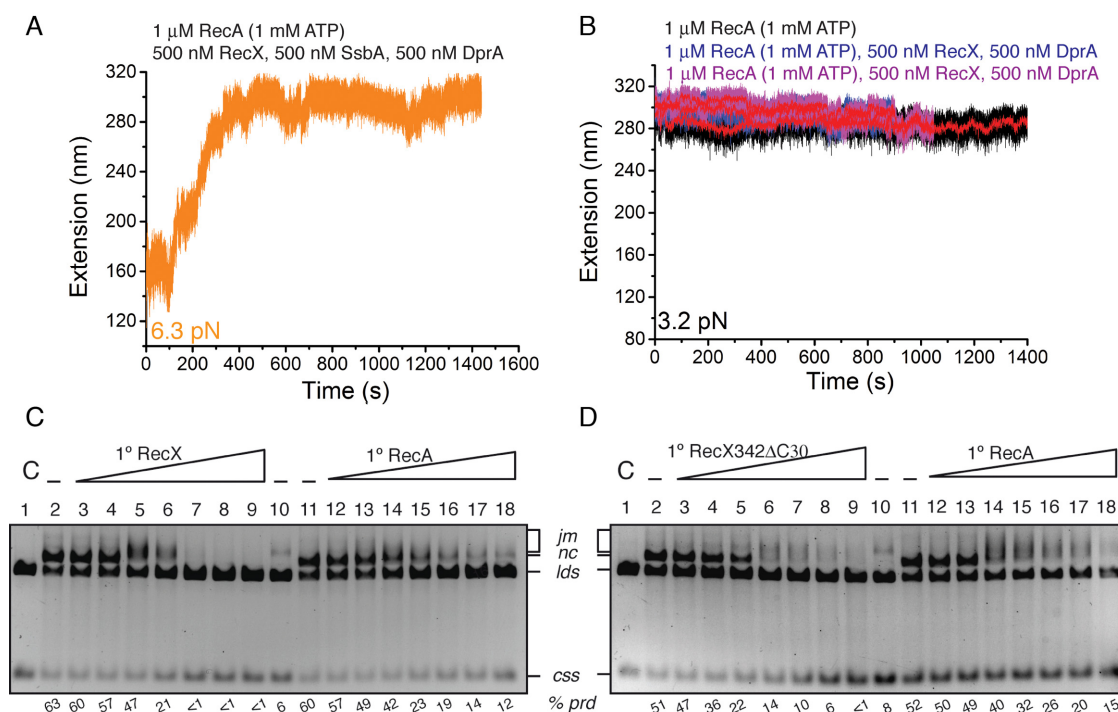
and dsDNA, followed by RecA-ATP (1 RecA/12 nt). In the presence of DprA-SsbA without RecX, RecA-ATP catalyzed DSE efficiently, with ~60% of the substrate converted to intermediate/product in 60 min (joint molecule [*jm*] plus nicked circular [*nc*]) (Figure 4C, lanes 2 and 11). Limiting concentrations of RecX (1:533 RecX:RecA molar ratio) were sufficient to impair accumulation of *nc* products (Figure 4C, lane 5). RecX at a 1:133 RecX:RecA molar ratio (~2 RecX/ssDNA molecule) completely abolished RecA-mediated *jm* formation (Figure 4C, lane 7). RecX<sub>Eco</sub> similarly abolishes RecA<sub>Eco</sub>-ATP-promoted *jm* formation at a 1:180 RecX<sub>Eco</sub>:RecA<sub>Eco</sub> molar ratio (27,29).

RecA-ATP polymerized on the SsbA-ssDNA-DprA complexes for 5 min, followed by variable amounts of RecX. At the time of RecX addition, ~8% of the substrate was converted to *jm* in 5 min (Figure 4C, lane 10). At RecX:RecA molar ratios of 1:266, *nc* products accumulated. Even at 1:32 RecX:RecA molar ratios reduced, but did not inhibit RecA-ATP-mediated DSE (Figure 4C, lane 18). RecA polymerized on the DprA-ssDNA-SsbA complex, after interaction with the homologous duplex, was activated to catalyze DNA pairing. Alternatively, in the presence of DprA-SsbA, RecA might catalyze DSE in a 3'→5' direction as described (17).

#### The absence of domains R3 $\alpha$ 1–3 do not compromise the RecX interaction with RecA

The ubiquitous *B. subtilis* RecX (264-amino-acid polypeptide) is distantly related to RecX<sub>Eco</sub> (~15% overall identity) or RecX<sub>Mtu</sub> (~20% overall identity) (8). RecX<sub>Eco</sub> (166-amino-acid polypeptide) is a modular protein consisting of three tandem repeats of  $\alpha$  three-helix motif (R1 $\alpha$ 1–3, R2 $\alpha$ 1–3 and R3 $\alpha$ 1–3) (30). RecX lacks the first two repeated domains (equivalent to RecX<sub>Eco</sub> R1 $\alpha$ 1 and R1 $\alpha$ 2). In RecX, however, the last 30 C-terminal residues might fold as three tandem  $\alpha$ -helix motifs, suggesting that it could also consist of seven  $\alpha$ -helix motif (R1 $\alpha$ 1, R2 $\alpha$ 1–3 and R3 $\alpha$ 1–3) (8). The *recX342* strain, which decreases interspecies recombination without significantly affecting the frequency of natural chromosomal transformation with homologous DNA, carried a single point mutation (L101P) in the first conserved  $\alpha$ -helix repeat (R1 $\alpha$ 1 domain) (8).

To map the RecX domain involved in the interaction with RecA we have tested whether RecX342 or a variant (RecX342 $\Delta$ C30) lacking the last 30 C-terminal residues (impaired in domain R1 $\alpha$ 1, and lacking domains R3 $\alpha$ 1–3) affected RecA-mediated DSE, we purified RecX342 and RecX342 $\Delta$ C30. As RecX342 was insoluble, only the soluble RecX342 $\Delta$ C30 was used for further analyses. When RecA was incubated with DprA-SsbA-ssDNA-RecX342 $\Delta$ C30 complexes, RecX342 $\Delta$ C30 inhibited RecA-mediated DSE with slightly greater efficiency than the wt protein (Figure 4D, lanes 5 and 6), but RecX342 $\Delta$ C30 did not inhibit RecA-ATP-mediated DSE when added to pre-formed SsbA-ssDNA-DprA-RecA complexes (Figure 4D, lanes 12–18). It is likely that RecA polymerized on the DprA-ssDNA-SsbA complexes (active state) competed partially with RecX or RecX342 $\Delta$ C30, and that impairment of R1 $\alpha$ 1 domain and the absence of domains R3 $\alpha$ 1–3 do not com-



**Figure 4.** DprA antagonizes the RecX inhibitory effect on RecA filament extension and strand exchange. (A) Typical extension time trace of RecA polymerized on ssDNA in the presence of a RecA, SsbA, RecX and DprA mixture. (B) Typical extension time traces of prefomed RecA filaments in the presence of RecA (black), RecA, RecX and DprA (blue, magenta) at  $\sim 3$  pN. (C and D) Homologous ssDNA and dsDNA were pre-incubated with SsbA, DprA and variable concentrations of RecX (C) or RecX342 $\Delta$ C30 (D) (0.3–25 nM) ( $1^\circ$  RecX or RecX342 $\Delta$ C30, lanes 3–9) or a fixed RecA (lanes 12–18) concentration ( $1^\circ$  RecA) (C and D, lanes 12–18) were added and the reaction incubated (60 min,  $37^\circ\text{C}$ ). Lane 1, DNA substrate controls (C); in lanes 2 and 11, RecX was omitted and in lane 10, the reaction was terminated after pre-incubation (5 min) in the absence of RecX or RecX342 $\Delta$ C30. Reactions were resolved by 0.8% agarose gel electrophoresis. Absence of RecX, –. Abbreviations: *css*, circular ssDNA; *lds*, linear dsDNA; *jm*, joint molecules intermediates; *nc*, nicked circular products. The positions of bands corresponding to *css*, *lds*, *cds*, *jm* and *nc* are indicated. Results are the mean ( $\pm 5\%$  SEM) of  $\geq 3$  independent experiments. Recombination product amount (*jm* + *nc*) is expressed as a percentage of total substrate added.

promise the RecX interaction with RecA (Figure 4C and D).

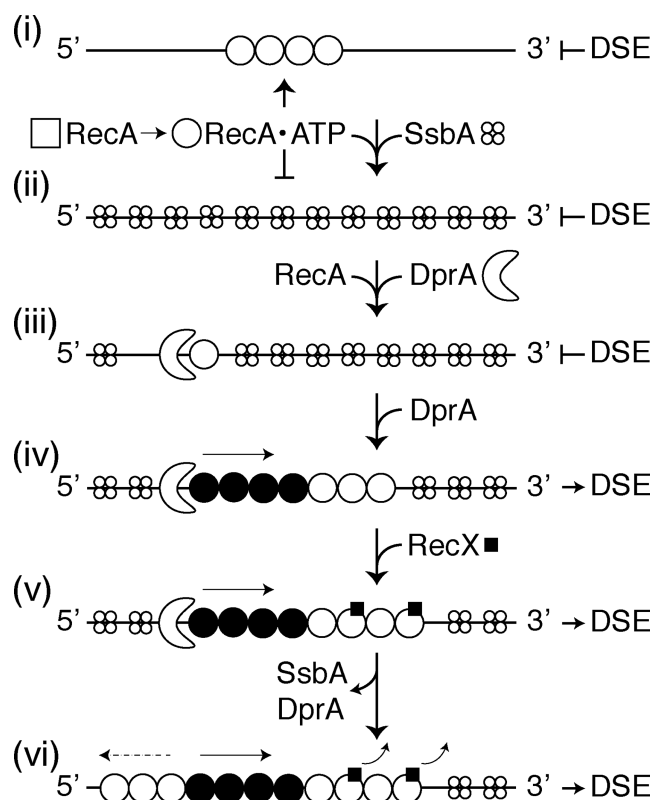
## DISCUSSION

In this report, we make four principal observations pertaining to RecA activities. First, the positive effect of DprA and the negative effect of RecX on RecA filament dynamics are crucial for chromosomal and plasmid transformation. Second, RecA, which is essential for chromosomal transformation and has no role in plasmid transformation, impairs plasmid transformation in the  $\Delta recX$  context. Third, RecX promotes RecA-ATP depolymerization with various disassembly kinetics, which suggests that passive filament capping at the 3'-end alone cannot explain these observations. Finally, RecA that is nucleated and polymerized on the DprA-ssDNA-SsbA complexes can counteract the negative effect of RecX, and this active RecA filament can catalyze DSE.

In all bacteria tested, the RecX protein regulates the activities of their cognate RecA (Figures 1–3) (27–30,46,50,51). To define the molecular mechanisms that control RecA filament growth during genetic recombination, we performed in-bulk and single-molecule experiments. Our data showed that RecA-ATP cannot nucleate or polymerize on the RecX-ssDNA, SsbA-ssDNA or SsbB-ssDNA

complexes, but RecA-dATP or RecA-ATP (at  $>10$  pN) can nucleate and polymerize on all these complexes. Stretching ssDNA by force reduces the conformational free energy cost,  $\Delta\Phi(f)$ , for RecA binding to ssDNA in a wide force range up to  $\sim 90$  pN, with maximum energy reduction at  $\sim 20$  pN (46). For forces of 10–50 pN, the conformational free energy cost is reduced by  $\geq 0.8 k_B T/nt$  ( $\geq 2.4 k_B T/\text{RecA monomer}$ ). ssDNA stretching by force, as well as force-independent RecA-dATP or RecA-ATP $\gamma$ S stabilizes the RecA NPF. A RecA stable filament thus antagonizes the inhibitory effects of RecX on RecA activities.

How RecX inhibits RecA activities and how RecA overcomes this effect are poorly understood. RecA-ATP cannot nucleate or polymerize on RecX-ssDNA, SsbA-ssDNA or SsbB-ssDNA complexes, although it can do so on SsbA-DprA-ssDNA-RecX complexes more efficiently than on DprA-ssDNA-RecX or SsbB-DprA-ssDNA-RecX complexes (Figure 1C and D). It is likely that RecA-ATP nucleated on DprA-ssDNA-SsbA complexes is less sensitive to RecX-mediated inhibition than RecA-ATP nucleated on DprA-ssDNA-SsbB complexes (Figure 1C and D). Indeed, RecA filaments formed in the presence of DprA-SsbA are stable when challenged with RecX (Figure 4A and B). We assumed that following interaction with SsbA, DprA, ssDNA and duplex DNA, RecA-ATP undergoes a transition



**Figure 5.** Model for RecA filament assembly in the DprA-ssDNA-SsbA complexes in the presence of RecX. Apo RecA (empty square) cannot nucleate on ssDNA. In the presence of ATP, RecA undergoes its first structural transition (square to circle) and can bind to incoming ssDNA (steps i). RecA-ATP cannot compete with the SsbA-ssDNA complexes (steps ii). DprA partially dislodges SsbB or SsbA. Like RecA-dATP, RecA-ATP interaction with DprA in the DprA-ssDNA-SsbA complex enhances assembly of RecA filaments (second transition stage, RecA-ATP, black circle) (steps iii and iv). DprA-SsbA activates RecA-ATP able to catalyze DSE. RecX blocks RecA assembly on ssDNA. DprA-SsbA makes a positive contribution to RecA-ATP assembly by enhancing its ability to displace RecX. Alternatively, dislodging DprA from the 3'-end might activate 3'→5' polymerization, which would be insensitive to RecX action (step v and vi). Positive (DprA-SsbA) and negative (RecX) effectors control the RecA polymerization/depolymerization dynamics.

that activates RecA to catalyze DSE, even when RecX was added to the reaction (Figure 4C, lanes 12–18). If RecX is present in the reaction mixture prior to RecA nucleation, however, this transition might not be possible and limiting RecX concentration (<2 RecX/ssDNA molecule) inhibits recombination (Figure 4C, lanes 3–9).

The molecular mechanisms that drive the order of protein addition and those by which RecX and DprA-SsbA control RecA activities during chromosomal and plasmid transformation are unclear. Based on previous data and the ones shown here, we propose that SsbA (or SsbA and SsbB) bound to the incoming ssDNA as soon as it leaves the entry channel. Apo-RecA cannot nucleate on ssDNA, and this barrier is overcome by ATP binding (Figure 5i). RecA-ATP cannot nucleate or polymerize on SsbA- or SsbB-coated ssDNA (Figure 5ii). SsbA and SsbB bind ssDNA with much higher affinity than DprA or RecA, suggesting that either SsbA or SsbB must be the first to bind incoming ssDNA

as soon as it leaves the entry channel (9,10). ssDNA-bound SsbA or SsbB recruits DprA, which in turn facilitates spontaneous SsbA or SsbB sliding along the ssDNA. ssDNA-bound DprA interacts with RecA (19,52) and enables limited RecA nucleation (Figure 5iii). RecA-ATP can nucleate on the DprA-ssDNA or SsbB-ssDNA-DprA complexes, but these ternary or quaternary complexes cannot mediate DSE (10). DprA interacts with SsbA and, by interacting with the first monomer of RecA, the SsbA-ssDNA-DprA complex might cap the 5'-end of the filament and activate RecA catalyzes DSE between circular ssDNA and a homologous duplex (Figure 5iv). The DprA-SsbA mediator promotes a structural change in RecA, decreases the free energy cost for RecA binding to SsbA-coated ssDNA, and contributes to RecA filament nucleation. When a RecA filament identifies a unique homologous sequence between incoming ssDNA and the duplex recipient, RecA mediates heteroduplex formation and catalyzes DSE to yields a chromosomal transformant (3).

It was shown that during transformation, RecA, DprA and RecX form mainly a discrete focus, RecA and DprA localize with the uptake apparatus at the cell pole and RecX is mostly at midcell in the nucleoid (7,8). Upon addition of DNA, RecA forms a thread that emanates from the uptake apparatus toward the nucleoid and ~80% of competent cells contain a RecX signal on the nucleoid (8). RecX, which co-localizes with RecA threads inhibits RecA filament extension (8), suggesting that RecX acts after SsbA-DprA-mediated RecA nucleation. Indeed, in exponentially growing cells the RecA threads persists for longer time in the absence of RecX (8). RecX might interact with discrete regions on the RecA filament and with the 3'-end, as shown for the RecA<sub>Eco</sub>-RecX<sub>Eco</sub> complex (28,30) (Figure 5, v). In our assays, slow depolymerization began presumably with RecX associated to the 3'-ends, which would prevent further addition of RecA monomers (3'-end capping model) (29). RecX in the ternary RecX-RecA-ssDNA complexes might nonetheless facilitate removal of a few internal RecA protomers, generating discontinuities in the RecA filament and more filament ends (Figure 5). The newly generated 5'-end facilitates more rapid RecA disassembly (active displacement model), as proposed (28,30), and the extent of RecA dissociation would be proportional to the discontinuities of the pre-assembled RecA filament (Figure 3). This is consistent with the observation that RecA undergoes depolymerization with various kinetics, and that the partially depolymerized RecA filament is able to repolymerize at higher forces (>7 pN) in the presence of RecX. In the absence of RecX, chromosomal and plasmid transformation decreased (Supplementary Table S1) and RecA threads are long-lived (8), which suggests that a long RecA filament (in the absence of RecX) might not form stable heteroduplexes and the search for homology would be unproductive.

DprA-SsbA interaction with RecA activates it to antagonize the inhibitory effect of RecX on RecA activities (Figure 5v and vi). After undergoing transition in the presence of DprA-SsbA, RecA-ATP might impede RecX interaction with the active RecA filament (especially at the 3'-end) and can catalyze DSE. Dynamic RecA assembly/disassembly might be a mechanism to modulate active RecA filament length during genetic recombination. Alternatively, RecA-



mediated stretching of the ssDNA facilitates spontaneous dislodgment of DprA–SsbA. In the absence of DprA, RecA might polymerize on the filament 5′-end, facilitating polymerization in the 3′→5′ direction, which is insensitive to RecX-mediated inhibition (Figure 5vi). This is consistent with the observation that RecA can catalyze strand exchange in either direction (5′→3′ or 3′→5′), but it shows a moderate preference (~3-fold) to initiate strand exchange at the 3′-end over the 5′-complementary end of the linear dsDNA substrate (17). Filament length would nonetheless be uncontrolled.

If RecA is recruited on heterologous ssDNA (plasmid DNA), DprA–SsbA activates a RecA-ATP filament that undergoes an unproductive homology search with the recipient chromosome. Interaction between two DprA proteins bound to complementary ssDNA (plasmid DNA) might facilitate strand annealing rather than RecA recruitment. Concomitantly, RecX promotes disassembly of preformed RecA NPF to terminate the unproductive search, indirectly promoting plasmid transformation. As discussed in the ‘Introduction’ section, the internalized complementary SsbA- (or SsbB)-coated plasmid strands are annealed by direct DprA–DprA interaction to reconstitute a circular replicon (15).

During recombinational repair, DprA is not expressed and the regulation of RecA filament assembly onto SSB-coated ssDNA requires RecFOR in *E. coli* and *B. subtilis* cells. In both bacteria, the RecFOR complex stimulates RecA nucleation and filament growth (16,17,22–26). Through direct interaction with RecA, RecX impedes RecA-ATP filament extension, leading to net passive or active RecA filament disassembly (Figure 3) (26–30). Finally, the RecFOR complex antagonizes the RecX negative effect (8,31). In contrast, lack of RecF, RecO or RecR protein does not affect natural chromosomal transformation in otherwise wt *B. subtilis* cells, indicating that the RecX can be antagonized through different pathways. The DprA–SsbA mediator indirectly reverses the RecX negative effect during genetic recombination.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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